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Validation of Pathway Analysis of Metals from Aged Dredged Material Using Plants and Worms

PURPOSE: Contaminants in dredged material (DM) placed in an upland situation such as a confined disposal facility (CDF) may move from substrates into food webs because of their contact with CDF-colonizing or CDF-inhabiting plants and animals, and as such may cause unacceptable risks outside the CDF. The primary goal of this technical note is to provide guidance on determining exposure-based bioaccumulation of metals and toxicological effects in test species representing two trophic levels.

BACKGROUND: Placement of DM in CDFs and its removal from CDFs for beneficial use require assessment of environmental risk. To this end the Decision-Making Framework and the U.S. Army Corps of Engineers (USACE)/U.S. Environmental Protection Agency (USEPA) Technical Framework may require exposure and effects assessments of relevant contaminant pathways prior to dredging. These assessments evaluate impacts on plants and animals in cases where terrestrial placement is selected as a disposal alternative, and there is no reason to believe that the DM is unacceptably contaminated. Currently no specific guidelines for contaminant residues in plants and animals exist. The purpose of this Dredging Operations and Environmental Research (DOER) Work Unit is to develop and validate methods or further validate existing methods to assess the potential for unacceptable environmental risk outside a CDF filled with contaminated DM, and to assess materials for their removal from an aged CDF and beneficial use.

INTRODUCTION: The USACE annually manages about 300 million cubic meters of DM. Historically about 50 percent is disposed of in open water, and 5 to 10 percent of the material that is unsuitable for open water disposal is placed into CDFs. USACE Districts are working to develop tools that help integrate topical quantitative information to yield quantifiable estimates of risk posed by dredged materials including uncertainty (Moore, Bridges and Cura 1998). The use of effects based testing and risk assessment is intended to supplement the analytical options currently available to DM managers by building on the existing technical framework (USEPA/USACE 1992) and the existing tiered approaches (USEPA/USACE 1991; USEPA/USACE 1998).

Several test organisms have been used by the USACE to evaluate bioaccumulation of metals from DM disposed in an upland situation (Folsom, Lee, and Bates 1981; Van Driel, Smilde, and Van Luit 1985; Folsom and Price 1989; Simmers et al. 1989). Unfortunately, in several of these studies the toxic effects of metals were sometimes noted but not quantified, nor were tests for toxic effects of organics performed. Based on a recent comparative study on the suitability of herbaceous plant and worm species as test organisms for DM from freshwater origin, *Cynodon dactylon* (bermudagrass) and *Eisenia fetida* (earthworm) were adopted (Best et al. 2001). The monocotyledonous *C. dactylon* was selected for its wide geographical distribution (common in North American regions south of Kentucky), rapid growth, profuse generative reproduction, and almost all biomass aboveground. The latter characteristic facilitates total biomass harvesting, and reduces sample number and analytical costs by a factor of two compared with species with extensive root systems, where shoots

and roots are usually harvested and analyzed separately. In addition, seeds germinate simultaneously within several days and the species can be cultivated in the testing environment. *C. dactylon* accumulates metals and selenium (Wong and Lau 1985; Wu, Huang, and Burau 1988). It accumulates metals to a lesser extent than *Cyperus esculentus* (yellow nutsedge), another plant used for bioaccumulation testing of metals in DM. For example, the tissue zinc (Zn) concentration in bermudagrass was 0.75 times the tissue Zn concentration in yellow nutsedge, with both species cultivated on the same DM (Best et al. 2001). The earthworm *E .fetida* was selected for its worldwide use and acceptance, facilitating comparison with bioaccumulation and toxicity data of other sites (Wilborn et al. 1997), and easy culturing in the laboratory. Toxicological effects in this test organism originate largely from direct skin contact with the toxic compounds in the interstitial water (American Society for Testing and Materials (ASTM) 1998; Kula and Larink 1998; Lokke and Van Gestel 1998). The worm has a litter-dwelling ecological strategy, lives in organic matter-rich soil, and reproduces via cocoons.

Based on a limited survey of upland CDFs of the USACE Districts, one wet site at Monroe, MI, was selected to serve as the DM source to be tested (Figure 1). This DM contained low levels of zinc (Zn), nickel (Ni), cadmium (Cd), lead (Pb), and vanadium (V), with Zn as the main contaminant (Best et al. 2001). The tests were performed on aged DM amended with metals-contaminated soil to enable extrapolation of the test results to field conditions.

The objectives of the current study were to evaluate the following in terrestrial plants and worms: (1) bioaccumulation of metals from metal-contaminated DM; (2) toxicity, as indicated by decreasing biomass on metal-contaminated DM; and (3) effects of substrate characteristics other than metal concentration on bioaccumulation and biomass.

MATERIAL AND METHODS

Bioaccumulation and Toxicity Assays. A dilution series was constructed by mixing contaminated DM with other soils. Subsequently, dose-response curves for Zn concentrations between 104 and 1943 mg Zn kg^{-1} DW were constructed for both plant and animal tests. A Zn concentration of 273 mg kg^{-1} substrate DW was considered representative for the Monroe CDF wet area. Control soils were used to verify performance of the test plants and worms.

The following responses were measured:

- For plants:
  - Accumulation, as measured by the plant tissue Zn accumulated in 55 days, in mg kg^{-1} DW (dry weight).
  - Toxicity, as measured by the plant biomass formed in 55 days, in g DW m^{-2}.
- For worms:
  - Accumulation, as measured by the worm tissue Zn accumulated in 28 days, in mg kg^{-1} DW.
  - Toxicity, as measured by the biomass of 12 worms present after 28 days, in g DW $cylinder^{-1}$.

Accumulation of metals other than Zn was also determined in plants and worms.

Figure 1. Monroe, MI, CDF from which the dredged material for the tests originated

Substrates. Two dredged materials from the Monroe, MI, CDF were excavated from the substrate surface to a depth of 0.33 m in June 2001: a moderately high Zn-contaminated material from a wet site and a low Zn-contaminated material from a dry site. The latter was used as a reference substrate (Best et al. 2001). The material was dried so that the moisture content was reduced to approximately 38 percent, riffled, mixed, and refrigerated. Selected DM was mixed with a highly Zn-contaminated soil from the Rock Island Arsenal, IL, Housing Area, to achieve the desired Zn concentrations (Table 1). Housing Area soil was dried and ground to pass a 2-mm sieve.

The substrate dilution series was constructed using four substrates: (1) DM from the Monroe CDF dry site, (2) DM from the Monroe CDF wet site, (3) soil from the Rock Island Housing Area, and (4) plant control (Table 1). A range of three Zn concentrations up to approximately 1,793 mg kg^{-1} DW was created by mixing the high-Zn substrate with a low-Zn substrate having characteristics

Table 1
Characteristics of dredged materials and soil used to create the substrate mixtures for the tests and control soils, mean values (N = 3)

Characteristic	Substrate					USEPA Guideline		Eastern United States
	Monroe-CDF Dry Site	Monroe-CDF Wet Site	Rock Island Soil	Plant Control	Invert. Control	CSCL Plants	CSCL Earth-worms	
Total Metals (mg kg^{-1} DW)								
Zinc	73.5	273.4	1936.7	18.2	<2.5	50	100	40
Nickel	14.9	57.2	<330	5.0	<2.0	30	200	11
Cadmium	0.48	2.04	BD	1.24	<2.0	4	20	—
Lead	49.0	70.9	1031.3	6.9	<5.0	50	500	14
Vanadium	9.6	53.2	BD	5.7	<4.0	2		43
DTPA-Extracted Metals (mg kg^{-1} DW)								
Zinc	ND	5.0	ND	4.5	0.19			
Nickel	ND	0.43	ND	0.12	<0.05			
Cadmium	ND	BD	ND	ND	ND			
Lead	ND	0.41	ND	0.74	0.32			
Vanadium	ND	0.09	ND	<0.05	<0.05			
Other								
pH_{water}	6.77	6.88	6.84	5.79	7.06			
Organic Matter (% DW)	4.09	8.90	5.46	76.29	1.33			
Dry Weight (% fresh wt.)	82.1	77.9	94.4	41.8	99.6			
Bulk Density (g DW mL^{-1})	1.46	1.29	2.07	1.27	1.12			

Note: Critical Stressor Concentration Levels in soil (CSCL) (USEPA 1999) based on screening benchmarks are given for comparison, and levels in the eastern U.S. are given for reference.
Abbreviations: ND = not determined; BD = below detection; DTPA = diethyltriamine-pentaacetic acid.

otherwise similar to the test substrate (dry Monroe CDF site) and with small quantities of the moderately high Zn substrate (wet Monroe CDF site). Organic matter (OM) concentrations were increased by adding small amounts of the organic matter-rich plant control soil in cases where concentrations higher than 5 percent OM were desired.

As a plant control substrate for plants, Baccto R Lite potting soil, Michigan Peat Company, Houston, TX, was used. This soil was alkalinized to the design initial pH of 6.8 (from pH 5.1) by spraying with 30 mL of 60.3 mg $CaCO_3$ L^{-1} per 15 L substrate. As a control substrate for worms, a standard artificial soil (Organization for Economic Cooperation and Development, Paris, France; Kula and Larink 1998) was used.

Plant Tests. The plant test used the following treatments:

- Zn concentration: four levels, i.e., reference (115), 123, 1,322, and 1,793 mg Zn kg^{-1} DW. A level of 1,500 mg Zn kg^{-1} DW is specified as being phytotoxic for most plants by Chaney (1983), and by Reeves, Baker, and Brooks (1995), but levels up to 5,000 mg Zn kg^{-1} DW have been found to be toxic for selected endemic grasses, according to Paschke, Redente, and Levy (2000).
- **Organic matter concentration:** two levels, i.e., 5 and 6 percent DW. Target organic matter levels were originally selected to represent the Monroe CDF dry site and Monroe CDF wet

site, with 5 and 9 percent OM, respectively, but the target levels were unfortunately not attained using this mixing procedure.

- **Moisture**: two levels, i.e., approximately 1/3 field capacity (15 percent) and close to field capacity (36 percent, field capacity being 38 percent). A moisture level at field capacity allows maximum mobility of metals in solution.
- **pH:** two target pH levels (6.8 and 5.4) were selected to represent the Monroe CDF sites and a highly organic (e.g., peat) soil. DM behaves more like surface soil as it ages, dewaters, and consolidates. It may also become enriched with organic matter when vegetative cover increases, and pH may drop to around 5.4 during this process. Mobility of metals increases with decreasing pH.

A control substrate served as a test to verify performance of the plants; four replicates at a 36 percent moisture level were tested. The plant study included a total of 132 units. For each unit, 0.207 g *C. dactylon* seeds were weighed, subjected to a germination-stimulating treatment, and placed on top of 1 L of the appropriate substrate mixture contained in 2-L plastic pots. Plants were allowed to grow for 55 days prior to harvesting. Germination was synchronized and stimulated by a factor of 2 by successively soaking each seed portion for 24 hr in 2 mL of 2 percent sodium hypochlorite ($NaClO_4$) in a refrigerator (5 ^oC) in darkness, and removing the hypochlorite by rinsing with demineralized water. *C. esculentus*, initially considered as another suitable candidate for a plant test species, was not used for the current tests, because attempts to enhance and synchronize germination of its tubers were unsuccessful.

Animal Tests. The animal test used the same treatments as the plant test, but was carried out at only one moisture level, 36 percent. The invertebrate study included a total of 68 units. For each unit, 12 *E. fetida* specimens were placed on top of 1 L of the appropriate substrate mixture contained in a 15-cm-diameter, 15 cm-high Plexiglas cylinder. Animals were allowed to grow for 28 days prior to harvesting.

Analyses. Prior to incubation each substrate mixture was analyzed in triplicate for chemical and physical characteristics. At the end of the incubation, fresh weight, dry weight and pH were determined in all substrate units. The total concentrations of Zn, Ni, Cd, Pb, and V were determined in all substrate mixtures (USEPA 1991). Bioavailable metals were also determined, using the diethyltriamine-pentaacetic acid (DTPA) extractable fraction as a measure (Lindsay and Norvell 1978). Moisture content was determined by drying at 105 °C in a forced-air oven until constant weight. Concentrations of organic matter were determined by loss on ignition at 550 °C, and bulk density volumetrically (Allen et al. 1974). pH_{KCl} was measured with a pH-meter (Beckman Model PHI40, Fullerton, CA) in a 1 M KCl solution in a fresh soil-to-liquid ratio of 1:2.5 (w/v). pH_{KCl} was converted to pH_{water} using a regression equation of $pH_{water} = 0.677 \times pH_{KCl} + 2.35$ (ISO 10390) (Best and Jacobs 2001).

Dry weight (plants and worms) was determined by drying the fresh material in a forced-air oven to constant weight (105 °C).The total metal concentrations were determined in all plant and animal samples, including Zn, Ni, Cd, Pb, and V, using a similar approach as for the substrates, but digesting 0.3 g DW.

Statistics. The STATGRAPHICS Plus for Windows 3 package (Manugistics, Rockville, MD, 1997) was used for statistical analyses.

Substrate mixtures were tested for significant differences in characteristics prior to incubation using Analysis of Variance (ANOVA). The level of significance was set at a 95 percent confidence level (p value ≤ 0.05). Control substrates were excluded from this test, because they merely served as verification for the viability of the test organisms. All substrate units were also tested for significant differences in pH after incubation using ANOVA.

Relationships between plant and worm responses and substrate metal concentrations were derived by regression. The p-value in the regression model is a measure of the significance of the regression coefficient; it was also set at a 95 percent confidence level (p value of ≤ 0.05). The R^2 value of the regression model indicates the proportion of the variance explained by the model; only regressions with R^2 values ≥ 0.50, i.e. explaining at least 50 percent of the variance in the data set, were considered as meaningful. Linear models provided a fit to both, the plant data and worm data.

The regression equations were used to predict tissue concentrations in plants and worms resulting from a specified soil concentration. The ratios between metal concentrations in the test organism and the concentration in the substrate, the Biota to Soil Accumulation Factor (BAF), were calculated also. These BAFs can be used in estimates of trophic transfer of contaminants from DM into plants and animals living in or visiting CDFs.

RESULTS AND DISCUSSION

Substrates. The eight substrate mixtures spanned a Zn concentration range of 104 to 1,943 mg kg^{-1} DW (Table 2). Unfortunately, the reference and lowest Zn concentrations in both the 5 and 6 percent organic matter series (mixes 1, 2, 5, and 6) were close (the analytical error for Zn was 2.8 percent). The concentrations of Ni, Cd, and Pb in the mixtures increased in concordance with Zn. Zn and Pb concentrations exceeded the Chemical Stressor Concentration Levels (CSCL) for plants and earthworms, while V concentrations exceeded the CSCLs only for plants. The latter implies that plants may have been stressed by the Zn, Pb, and V concentrations in the substrates, while earthworms were affected only by those of Zn and Pb. The mixtures differed significantly in the total and DTPA-extractable concentrations of Zn, Ni, Cd, and Pb, and an interaction was found between organic matter content and the total- and DTPA-extractable concentrations of Zn, Ni, and Pb. pH values in the substrates after incubation with plants and animals showed no significant differences (average values were 7.37 with plants and 7.06 with animals).

Bioaccumulation and Toxicity in Plants. In the plant material only Zn and Ni were recovered, while other metals were below detection. Tissue Zn concentration increased with increasing substrate Zn concentration (Table 3). Plant biomass at the highest substrate Zn concentration was insufficient for metal analysis. Plant biomass decreased with increasing substrate Zn concentration (Table 3).

The relationships between the plant responses and the metal concentrations, organic matter concentrations, and moisture contents in the substrate mixtures were derived using regression techniques.

Table 2
Characteristics of the substrate mixtures prior to incubation

Characteristic	Substrate Mixture							
	5%OM				6%OM			
	Ref-Zn (115) Mix 1	Zn 123 Mix 2	Zn 1322 Mix 3	Zn 1793 Mix 4	Ref-Zn (115) Mix 5	Zn 123 Mix 6	Zn 1322 Mix 7	Zn 1793 Mix 8
Total Metals (mg kg^{-1} DW)								
Zinc	104.3	124.7	1300.0	1673.3	124.7	120.3	1343.3	1913.3
Nickel	21.2	23.7	36.3	43.5	23.6	24.4	37.5	45.5
Cadmium	<3.92	<3.92	3.95	5.92	<3.92	<3.92	4.6	6.59
Lead	55.7	51.5	682.7	832.3	51.9	48.9	526.3	1766.7
Vanadium	9.3	16.5	11.9	18.4	13.2	15.2	11.2	16.5
DTPA-Extracted Metals (mg kg^{-1} DW)								
Zinc	24.8	25.0	289.7	305.3	22.7	29.0	287.3	369.3
Nickel	2.54	2.33	4.89	10.84	2.35	1.91	4.93	5.79
Cadmium	0.21	0.23	1.53	2.91	0.2	0.24	1.51	2.08
Lead	16.6	15.2	235.0	282.0	15.7	14.5	203.7	203.7
Vanadium	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Other								
pH_{water}	6.79	6.90	6.87	6.83	6.97	6.98	6.95	6.90
Organic Matter (% DW)	4.28	5.14	5.41	5.39	5.45	6.13	5.97	6.13
Dry Weight (% fresh wt.)	81.9	81.7	89.7	93.0	79.6	80.2	88.3	92.4
Bulk Density (g DW mL^{-1})	1.37	1.51	1.43	1.43	1.40	1.24	1.48	1.31

Note: Ref-Zn (reference Zn) concentration and Zn concentrations in mg kg^{-1} DW. Target levels of pH, moisture, and nutrients were established gradually after initiation of the experiment. Mean values (N = 3).

Table 3
Responses of *C. dactylon* after 55-day incubation

Zn Concentration (mg kg^{-1} DW)	[Tissue-Zn] Plant (mg kg^{-1} DW)	Total Plant Biomass (g DW m^{-2})
115	123.07 ± 11.97 A	8.924 ± 5.208 A
123	140.79 ± 35.29 B	5.246 ± 3.899 B
1322	578 ± 578 C	0.101 ± 0.169 C
1793		0.005 ± 0.010 C

Note: Mean values and standard deviations (N = 4). Different letters indicate statistically significant differences between means at the 95 percent confidence level, according to Fisher's least significant difference procedure

The equations describing the linear regressions that fit the data are presented in Table 4. The tissue Zn concentration and biomass of the plants could be predicted from the total and DTPA-extractable Zn concentrations in the substrate, without any of the other substrate parameters contributing to the model (Table 4), using the criterion that only regression equations with a significant fit at the 95 percent confidence level ($p \leq 0.05$) that explained at least 50 percent of the variance in the data set ($R^2 \geq 0.50$) were considered as meaningful. Predictions of tissue Zn concentration ($R^2 = 0.83$) were more accurate than those of plant biomass ($R^2 = 0.50$). No significant relationships between substrate parameters and plant biomass and tissue Ni concentration were found.

Table 4
Relationships between plant responses and metal concentrations in the substrate mixtures

Response	Statistic Parameter			Statistic Fitted Model	
	Estimated Value	Standard Error	p-value	p-value	R^2
[Total-Zn] substrate					
[Tissue-Zn] plant*				<0.001	0.83
a	88.9425	4.8349	<0.001		
b	0.3645	0.0225	<0.001		
Plant biomass**				<0.001	0.50
a	7.4390	0.4773	<0.001		
b	-0.0046	0.0004	<0.001		
[DTPA-Extractable-Zn] substrate					
[Tissue-Zn] plant*				<0.001	0.83
a	88.6537	4.8578	<0.001		
b	1.7035	0.1056	<0.001		
Plant biomass**				<0.001	0.50
a	7.6161	0.4760	<0.001		
b	-0.0239	0.0021	<0.001		

Note: Linear models provided the fit, being Y = a + bX, in which Y is the plant response and X the substrate metal concentration.
* Values for metal concentrations in mg kg^{-1} DW
** Values for plant biomass in g DW m^{-2}

Frequently used measures to describe toxicity are the lethal concentration (LC), i.e., the concentration of a toxin that kills a specified percentage of the organism; the effective concentration (EC), i.e., the concentration of a toxin that produces an observable negative effect in the organism; and the phytotoxicity threshold (PT), i.e., the tissue concentration of a plant that corresponds with a defined growth reduction (ASTM 1994; ASTM 1998).

The effective concentration that reduced plant biomass by 50 percent in 55 days (55d-EC50) for Zn was calculated by substituting 50 percent of the maximum plant biomass value for Y in the equation describing the relationship between tissue Zn concentration and total Zn concentration in the substrate (Table 4). Thus, a 55d-EC50 of 645 mg total-Zn kg^{-1} substrate DW was found. This EC50 is far higher than the CSCL for Zn in plants of 50 mg kg^{-1} DW.

The tissue Zn concentrations associated with normal growth and phytotoxicity, respectively, were calculated by substituting the relevant total Zn substrate concentrations in the equation describing the relationship between tissue Zn concentration and total Zn concentration in the substrate (Table 4). The total Zn concentration in the substrate was included in the calculations because it enables comparison with literature values. A tissue Zn concentration of 163 mg kg^{-1} DW was associated with normal growth allowing plants to be green and vigorous, and producing a considerable amount of biomass. It was found by substituting the total Zn concentrations of the four substrates with the lowest Zn levels (204 mg kg^{-1} substrate DW on average) in the equation. The phytotoxic tissue Zn concentration was calculated similarly, by substituting the 55d-EC50 substrate concentrations in the regression. Thus, a phytotoxic tissue Zn concentration of 324 mg kg^{-1} DW was found. The tissue Zn concentrations associated with normal and phytotoxic growth, respectively, are in the same order of magnitude as those found for agricultural crops and grass varieties (Boawn and Rasmussen 1971;

Davis and Beckett 1978; Best, Geter, and Larson in preparation), but far higher phytotoxic tissue Zn concentrations have been found for endemic grass species (Paschke, Redente, and Levy 2000).

The BAF for Zn in *C. dactylon*, calculated using the linear regression equation of Table 4, decreased from 1.25 to 0.43 between 100 and 1,322 mg total-Zn kg^{-1} substrate (Table 5).

Table 5
Bioassay tissue metal concentrations and BAF calculated using the regression equations relating plant and worm responses to total metal concentration in the substrate (Table 4 for plants, Table 7 for worms)

Bioassay	*C. dactylon*		*E. fetida*	
[Total metal] Substrate (mg kg^{-1} DW)	[Tissue metal] (mg kg^{-1} DW)	BAF	[Tissue metal] (mg kg^{-1} DW)	BAF
Zinc				
100	125.4	1.25	92.9	0.93
1,000	453.5	0.45	141.0	0.14
1,322	571.0	0.43	158.2	0.12
1,793			183.3	0.10
Lead				
100			13.9	0.14
1,000			42.6	0.04
1,300			52.1	0.04

Bioaccumulation and Toxicity in Worms. In the animal material Zn, Ni, Cd, and Pb were recovered. The 28-day *E. fetida* tissue-Zn concentration increased with the substrate Zn concentration (Table 6). No growth inhibition was noted. The tissue concentrations of Ni and Pb covaried with those of Zn.

Table 6
Responses of *E. fetida* after 28-day incubation

Zn Concentration (mg kg^{-1} DW)	[Tissue-Zn] Worm (mg kg^{-1} DW)	[Tissue-Pb] Worm (mg kg^{-1} DW)	Total Worm Biomass (g DW $Cylinder^{-1}$)
115	93.44 ± 6.29 A	6.03 ± 1.91 A	0.902 ± 0.098 A
123	91.81 ± 10.86 A	6.68 ± 2.93 A	0.820 ± 0.148 AB
1,322	167.19 ± 6.08 B	43.49 ± 17.36 B	0.975 ± 0.131 BC
1,793	176.94 ± 47.17 B	50.76 ± 18.56 B	0.981 ± 0.094 C

Note: Mean values and standard deviations (N = 4). Different letters indicate statistically significant differences between means at the 95 percent confidence level, according to Fisher's least significant difference procedure

The relationships between the animal responses and the metal and organic matter concentrations were derived using regression techniques. The equations describing the regressions that fit the data are presented in Table 7. The animal tissue Zn concentration could be accurately predicted from the total Zn and DTPA-extractable Zn concentrations in the substrate, and the tissue Pb concentration from the total Pb and DTPA-extractable Pb concentrations in the substrate (Table 7). Regression models relating tissue metal concentrations to DTPA-extractable metal concentrations in the substrate usually explained a larger part of the variability in the data set than those relating tissue

Table 7
Relationships between worm responses and metal concentrations in the substrate mixtures

Response	Statistic Parameter			Statistic Fitted Model	
	Estimated Value	Standard Error	p-value	p-value	R^2
[Total-Zn] Substrate					
[Tissue-Zn] worm*				<0.001	0.68
a	87.5731	5.1617	<0.001		
b	0.0534	0.0046	<0.001		
Worm biomass**				0.005	0.12
a	0.8859	0.0224	<0.001		
b	5.796×10^{-5}	2.002×10^{-5}	0.005		
[DTPA-Zn] Substrate					
[Tissue-Zn] worm*				<0.001	0.70
a	85.9351	5.1291	<0.001		
b	0.2744	0.0229	<0.001		
Worm biomass**				0.005	0.12
a	0.8842	0.0227	<0.001		
b	29.7×10^{-5}	10.1×10^{-5}	0.004		
[Total-Pb] Substrate					
[Tissue-Pb] worm*				<0.001	0.56
a	10.7109	2.6942	<0.001		
b	0.0319	0.0035	<0.001		
Worm biomass**				0.020	0.08
a	0.9022	0.0202	<0.001		
b	6.423×10^{-5}	2.677×10^{-5}	0.020		
[DTPA-Pb] Substrate					
[Tissue-Pb] worm*				<0.001	0.68
a	4.5737	2.5881	0.082		
b	0.1798	0.0156	<0.001		
Worm biomass**				0.007	0.11
a	0.8876	0.0223	<0.001		
b	38.054×10^{-5}	13.512×10^{-5}	0.007		

Note: Linear models provided the fit, being Y = a + bX, in which Y is the worm response and X the substrate metal concentration.
* Values for metal concentrations in mg kg^{-1} DW
** Values for worm biomass in g DW $cylinder^{-1}$

metal concentrations to total metal concentrations in the substrate (Table 7). The tissue Zn concentrations in *E. fetida*, ranging from 92 to 177 mg Zn kg^{-1} DW, were relatively high, and apparently not regulated within a narrow range around 120 mg kg^{-1}, as found by Lock and Janssen (2001). Animal biomass could not be accurately predicted from any of the metal concentrations in the substrate, since all regression models explained only a minor part of the variability in the data set (for total- and DTPA-extractable Zn concentration, $R^2 \leq 0.12$; Table 7). Animal growth, i.e., the increase in biomass, was also tested, but no significant treatment effects were noted. None of the substrate characteristics other than Zn concentration and Pb concentration affected animal biomass and tissue metal concentration.

It was not possible to calculate an accurate lethal concentration that killed 50 percent of the animals in 28 days (28d-LC50) or that reduced animal biomass by 50 percent (28d-EC50), for *E. fetida* in the current study, because the animal mass still increased in animals exposed to the highest Zn dose.

It is possible that doses of 1,793 mg Zn kg DW^{-1} may cause a reduction in growth. Most ecotoxicity tests use spiked soils that have not been aged long enough to allow the metal to reach equilibrium. In one study on zinc toxicity in aged soil (Smit and Van Gestel 1996), it was found that toxicity is far lower in aged than in spiked soils and that the EC50 for zinc is 1,749 mg kg^{-1} DW. The latter value is in the same range as found in the current study (1,793 mg kg^{-1} DW). All these EC50 values are far higher than the CSCL for Zn in invertebrates of 100 mg kg^{-1} DW.

The BAFs for Zn and Pb over the metal concentration ranges spanned by the substrate mixtures tested were calculated using the regression equations of Table 7, referring to total metal concentrations in the substrate. The BAF for Zn in *E. fetida* decreased from 0.93 to 0.10 between 100 and 2,000 mg total Zn kg^{-1} substrate DW, and for Pb from 0.14 to 0.04 between 100 and 2,000 mg total Pb kg^{-1} substrate DW (Table 5).

CONCLUSIONS: Results of this study revealed the following. Bermudagrass responded to increasing Zn levels by exhibiting increased bioaccumulation and toxicity. The relatively low effective concentration that reduces plant biomass by 50 percent in 55 days (55d-EC50) of 645 mg total Zn kg^{-1} substrate DM for bermudagrass may indicate that this species is too sensitive to accurately project metal bioaccumulation at high Zn levels. Bermudagrass may be used for bioaccumulation and toxicity assessment at lower Zn levels, e.g., for beneficial use of DW, in the southern part of North America up to Kentucky, but not in more northern U.S. regions. DTPA-extracted metal concentrations were accurate predictors of Zn bioaccumulation in bermudagrass and worms, and DTPA extraction of metals appears to be a good screen in tiered risk assessment.

Earthworms did not exhibit metal toxicity in the range studied. A correlation was found between bioaccumulation and substrate Zn levels. Therefore, earthworm bioaccumulation may be a valid Tier III assay.

USEPA CSCLs were found to be far lower than the substrate metal concentrations at which adverse effects in bermudagrass were detected, and may be considered as conservative rather than realistic. Additional representative grass species may be considered in future efforts to attain broader geographical representation and application of test methods.

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